

# The selection of buffers for UV monitoring at 206 nm

**Roger Bishop  
Hans Lundin**

*LKB-Produkter AB  
S-161 25 Bromma  
Sweden*

**Instrument**

LKB 2138 Uvicord S UV Monitor

**Field of application**

gel filtration  
ion-exchange, adsorption and  
affinity chromatography  
density gradient centrifugation

**Date**

Dec. 1978

## INTRODUCTION

Buffers are used in several protein separation techniques (gel filtration; ion-exchange, adsorption and affinity chromatography; density gradient centrifugation) in order to maintain a suitable pH environment, since the configuration of the amino acid backbone, and therefore the degree of activity of proteins such as enzymes, hormones and hormone receptors, is pH-dependent. Enzymes, for example, are usually active only within a pH range of 0.2 to 1.0 pH units.

By far the most-often used detector for protein separation techniques is the ultraviolet (UV) monitor, since it is more specific and more sensitive than, say, the refractometer, and its construction can also be rather simple. Most fixed-wavelength UV monitors operate at one or

both of the wavelengths 254 nm and 280 nm, the latter being used for monitoring proteins.

The monitoring wavelength 206 nm, which on fixed-wavelength monitors is available only with LKB 2138 Uvicord S and the now-discontinued LKB 2089 Uvicord III, has distinct advantages over the two 'conventional' UV wavelengths. Generally speaking, any compound which contains a carboxyl group, an ester linkage, or an amide or peptide bond will have an absorption maximum between 200 nm and 210 nm, and can be detected at 206 nm. In practice, proteins can be monitored at a much higher sensitivity at 206 nm than at 280 nm: for example, the sensitivity for albumin is 40 times greater at 206 nm than at 280 nm, and for other proteins the increase in sensitivity is 2 to 200 times. By monitoring at 206 nm, the

biochemist can also detect substances in the classes amino acids, peptides, fatty acids, sterols, and carbohydrates; all of these have very low or no absorbance at the 'conventional' wavelengths.

Some of the buffers commonly used in column chromatography also contain functional groups which absorb at 206 nm. It is therefore obvious that more consideration of the nature of the buffer (and its purity) should be taken when monitoring chromatographic separations at 206 nm than is necessary when monitoring them at 254 nm or 280 nm. This Application Note describes which of the commonly-used buffers are suitable for monitoring at 206 nm without changing the monitoring parameters, and how buffers which absorb at 206 nm can still be used by judicious choice of flow cell and absorbance range of Uvicord S.

## EXPERIMENTAL

### Buffer components

The following buffer components were examined for their suitability for monitoring at 206 nm. In order to avoid absorbance by impurities, only Analar® or other high-purity grades were used.

#### Phosphoric acid, $\text{H}_3\text{PO}_4$

$\text{pK}_1 = 2.15$ ;  $\text{pK}_2 = 7.20$ ;  $\text{pK}_3 = 12.38$

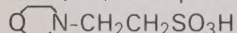
#### Citric acid

$\text{pK}_1 = 3.13$ ;  $\text{pK}_2 = 4.76$ ;  $\text{pK}_3 = 6.40$

#### Acetic acid

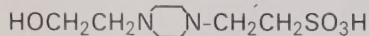
$\text{pK} = 4.76$

#### MES, 2-(*N*-morpholino)ethanesulphonic acid



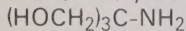
$\text{pK} = 6.15$

#### HEPES, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid



$\text{pK} = 7.55$

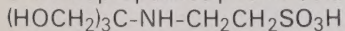
#### TRIS, tris(hydroxymethyl)aminomethane



$\text{pK} = 8.3$

#### TAPS, *N*-tris(hydroxymethyl)methyl-

3-aminopropanesulphonic acid



$\text{pK} = 8.55$

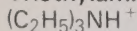
#### Boric acid, $\text{H}_3\text{BO}_3$

$\text{pK} = 9.24$

#### Ammonium, $\text{NH}_4^+$

$\text{pK} = 9.25$

#### Triethylammonium



$\text{pK} = 10.72$

The one or several  $\text{pK}$  value(s) stated for these substances indicate the  $\text{pH}$ (s) where the maximum buffering capacity occurs. To obtain a satisfactory buffering capacity, the substance should not be used at more than 1  $\text{pH}$  unit either side of the  $\text{pK}$  value.

As with any biological system, it should be ascertained that the buffer in use does not react with the protein being purified or with any essential component. For example, some buffers can remove metal ions essential

for enzyme activity; buffers containing carboxylic acids or inorganic phosphate may be substrates or inhibitors of enzymes; borate is well-known for forming complexes with a number of metabolites, such as carbohydrates.

## Uvicord S UV Monitor

The features of LKB Uvicord S UV Monitor which are pertinent to this Application Note are the lamp and interference filter for monitoring at 206 nm, the ABS-RANGE switch for selecting absorbance ranges from 0.005A to 2A, the ABS BASELINE ADJUST control, and the range of flow cells with optical pathlengths from 0.5 mm to 5 mm (the standard cell has a rectangular cross-section and can be used for optical pathlengths 2.5 mm or 5 mm). Absorbance is expressed in terms of absorbance units, A, where  $A = -\log(I/I_0)$ .

In the simplest case, buffer absorbance at 206 nm can be suppressed by up to 0.4A when the standard 2.5 mm-optical pathlength flow cell is used. This is done simply by means of the ABS BASELINE ADJUST control.

In case where the buffer absorbance exceeds 0.4A, a flow cell with a shorter optical pathlength can be used, and the monitoring sensitivity maintained by switching to a more sensitive absorbance range. For example, a reduction of optical pathlength from 2.5 mm to 0.5 mm will give a five-fold increase in buffer absorbance which can be suppressed, and the sensitivity can be maintained by switching to a five times as sensitive absorbance range, for example, from 0.5A to 0.1A.

## METHODS

The buffers described in the following sections were prepared as indicated in standard biochemical reference books (Ref. 1, 2). The buffers represent common ones used for each technique. For certain buffers, the absorbance was recorded for solutions at the extremes of the  $\text{pH}$  range and in the middle of the range.

In all the experiments, the absorbance was monitored with flowing buffer, using the standard 2.5 mm-optical pathlength flow cell of LKB Uvicord S UV monitor and the 206 nm lamp and interference filter. The absorbance figures given may differ slightly between instruments because of slight differences in the pathlengths of the cells.

## Gel filtration

- 0.25 N  $\text{H}_3\text{PO}_4$  adjusted to  $\text{pH}$  2.04 with triethylamine; absorbance = 0.015.  
0.25 N  $\text{H}_3\text{PO}_4$  adjusted to  $\text{pH}$  3.2 with triethylamine; absorbance = 0.030.  
In neither case is adjustment of the monitoring parameters necessary.
- Sodium acetate/acetic acid buffer solutions,  $\text{pH}$  approx. 3.7-5.6. The buffer solutions are prepared by mixing  $x$  ml 0.2 M-NaOAc with  $y$  ml 0.2 M-HOAc (see Table 1).

At acetate concentrations of 0.2 M, the buffer absorbances at the  $\text{pH}$  values tested are too high ( $> 0.4$  A) to be completely suppressed. The two buffer components were therefore each diluted to 15, 20 and 30 mM and new buffer solutions prepared for each of these concentrations; the  $\text{pH}$  of

Table 1\*

Sodium acetate/acetic acid buffer solutions: preparation, and absorbance at 206 nm

x ml 0.2 M-NaOAc and y ml 0.2 M-HOAc are mixed. Absorbance measured in cell with 2.5 mm-optical pathlength.

| pH, 18°C | x ml<br>0.2 M-NaOAc | y ml<br>0.2 M-HOAc | Measured<br>absorbance |
|----------|---------------------|--------------------|------------------------|
| 3.7      | 10.0                | 90.0               | 1.16                   |
| 3.8      | 12.0                | 88.0               |                        |
| 4.0      | 18.0                | 82.0               |                        |
| 4.2      | 26.5                | 73.5               |                        |
| 4.4      | 37.0                | 63.0               |                        |
| 4.6      | 49.0                | 51.0               | 1.62                   |
| 4.8      | 59.0                | 41.0               |                        |
| 5.0      | 70.0                | 30.0               |                        |
| 5.2      | 79.0                | 21.0               |                        |
| 5.4      | 86.0                | 14.0               |                        |
| 5.6      | 91.0                | 9.0                | 1.75                   |

\*From H.A. McKenzie in Data for Biochemical Research, 2nd. ed. (1969), p. 487, ed by R.M.C. Dawson et al. Reproduced with the permission of Oxford University Press.

each solution was checked and agreed with the value expected.

Figure 1 shows the effect of pH on the absorbance at each concentration. Only the 15 mM buffer solution has an absorbance of less than 0.4 A over its whole pH range.

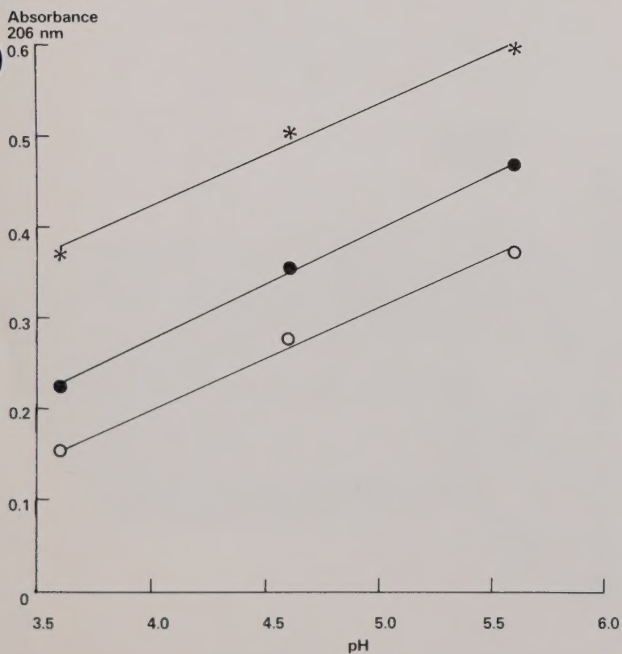


Fig. 1: Absorbance at 206nm of sodium acetate/acetic acid buffer solutions as a function of pH. The buffer solutions were prepared using the volumes indicated in Table 1, but with initial NaOAc and HOAc concentrations of 15 mM (o-o-o), 20 mM (●-●-●) and 30 mM (★-★-★).

3. Sodium phosphate buffer solutions, pH approx. 5.8-8.0. The buffer solutions are prepared by mixing 0.2 M- $\text{Na}_2\text{HPO}_4$  with 0.2 M- $\text{NaH}_2\text{PO}_4$  (see Table 2).

Table 2\*

Sodium phosphate buffer solutions: preparation, and absorbance at 206 nm

x ml 0.2 M- $\text{Na}_2\text{HPO}_4$  and y ml 0.2 M- $\text{NaH}_2\text{PO}_4$  mixed, then diluted to 100 ml with water. Absorbance measured in cell with 2.5 mm-optical pathlength.

| pH, 25°C | x ml<br>0.2 M- $\text{Na}_2\text{HPO}_4$ | y ml<br>0.2 M- $\text{NaH}_2\text{PO}_4$ | Measured<br>absorbance |
|----------|------------------------------------------|------------------------------------------|------------------------|
| 5.8      | 4.0                                      | 46.0                                     | 0.10                   |
| 6.0      | 6.15                                     | 43.85                                    |                        |
| 6.2      | 9.25                                     | 40.75                                    |                        |
| 6.4      | 13.25                                    | 36.75                                    |                        |
| 6.6      | 18.75                                    | 31.25                                    |                        |
| 6.8      | 24.5                                     | 25.5                                     | 0.30                   |
| 7.0      | 30.5                                     | 19.5                                     |                        |
| 7.2      | 36.0                                     | 14.0                                     |                        |
| 7.4      | 40.5                                     | 9.5                                      |                        |
| 7.6      | 43.5                                     | 6.5                                      |                        |
| 7.8      | 45.75                                    | 4.25                                     |                        |
| 8.0      | 47.35                                    | 2.65                                     | 0.45                   |

\*From H.A. McKenzie in Data for Biochemical Research, 2nd. ed. (1969), p. 489, ed. by R.M.C. Dawson et al. Reproduced with the permission of Oxford University Press.

Since the absorbances obtained when 0.2 M solutions were mixed exceeded 0.4 A, the buffer component solutions were diluted to 0.1 M and the same volumes mixed and diluted to 100 ml. Figure 2 indicates the absorbance as a function of pH obtained when the 0.2 M solutions are used to prepare the buffer solutions, and when the 0.1 M solutions are used. In the latter case, buffer absorbance can be suppressed over the whole pH range.

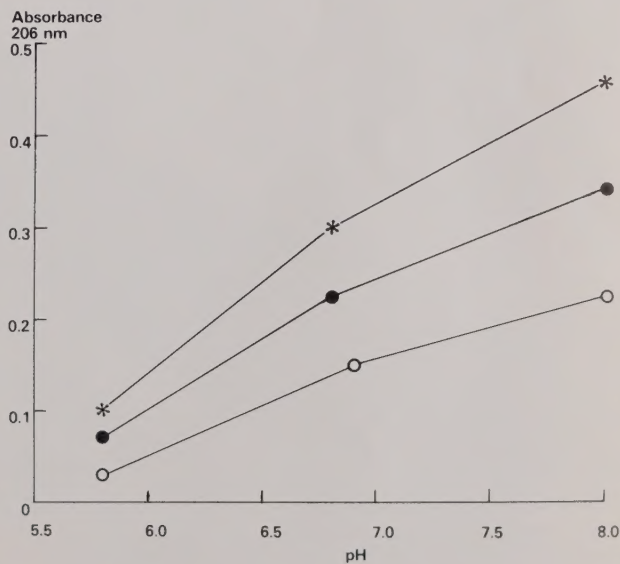


Fig. 2: Absorbance at 206 nm of sodium phosphate buffer solutions and Clark & Lubs buffer solutions, 5.8-8.0, as a function of pH. The sodium phosphate buffer solutions were prepared using the volumes indicated in Table 2, with initial concentrations of 0.1 M (●-●-●) and 0.2 M (★-★-★). The Clark & Lubs buffer solutions (o-o-o) were prepared as described in Table 3.

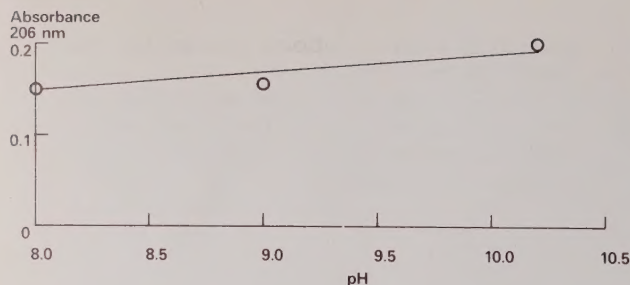


Fig. 3: Absorbance at 206 nm of Clark & Lubs buffer solutions were prepared as described in Table 4.

4. Clark and Lubs solutions, pH 5.8-8.0. The buffer solutions are prepared by mixing 0.1 M-KH<sub>2</sub>PO<sub>4</sub> with 0.1 M-NaOH (see Table 3).

Table 3\*

Clark and Lubs solutions, pH 5.8-8.0: preparation, and absorbance at 206 nm

50 ml 0.1 M-KH<sub>2</sub>PO<sub>4</sub> mixed with x ml 0.1 M-NaOH and diluted to 100 ml with water. Absorbance measured in cell with 2.5 mm-optical pathlength.

| pH, 25°C | x    | Buffer value, β | Measured absorbance |
|----------|------|-----------------|---------------------|
| 5.80     | 3.6  |                 | 0.04                |
| 5.90     | 4.6  | 0.010           |                     |
| 6.00     | 5.6  | 0.011           |                     |
| 6.10     | 6.8  | 0.012           |                     |
| 6.20     | 8.1  | 0.015           |                     |
| 6.30     | 9.7  | 0.017           |                     |
| 6.40     | 11.6 | 0.021           |                     |
| 6.50     | 13.9 | 0.024           |                     |
| 6.60     | 16.4 | 0.027           |                     |
| 6.70     | 19.3 | 0.030           |                     |
| 6.80     | 22.4 | 0.033           | 0.15                |
| 6.90     | 25.9 | 0.033           |                     |
| 7.00     | 29.1 | 0.031           |                     |
| 7.10     | 32.1 | 0.028           |                     |
| 7.20     | 34.7 | 0.025           |                     |
| 7.30     | 37.0 | 0.022           |                     |
| 7.40     | 39.1 | 0.020           |                     |
| 7.50     | 40.9 | 0.016           |                     |
| 7.60     | 42.4 | 0.013           |                     |
| 7.70     | 43.5 | 0.011           |                     |
| 7.80     | 44.5 | 0.009           |                     |
| 7.90     | 45.3 | 0.008           |                     |
| 8.0      | 46.1 |                 | 0.22                |

\*From H.A. McKenzie in Data for Biochemical Research, 2nd. ed. (1969), p. 490, ed. by R.M.C. Dawson et al. Reproduced with the permission of Oxford University Press.

It can be seen from this Table and also from Figure 2 that none of the buffer solutions has an absorbance exceeding 0.4 A, and can therefore be used without change.

5. Clark and Lubs solutions, pH 8.0-10.2. The buffer solutions are prepared by mixing a solution containing 0.1 M-KCl and 0.1 M-H<sub>3</sub>BO<sub>3</sub> with a solution of 0.1 M-NaOH (see Table 4).

In this case, too, each buffer solution has an absorbance of less than 0.4 A, and can therefore be used without change. Figure 3 shows the very small

Table 4\*

Clark and Lubs solutions, pH 8.0-10.2: preparation, and absorbance at 206 nm.

50 ml of a mixture of 0.1 M-KCl and 0.1 M-H<sub>3</sub>BO<sub>3</sub> mixed with x ml 0.1 M-NaOH, then diluted to 100 ml with water.

| pH, 25°C | x    | Buffer value, β | Measured absorbance |
|----------|------|-----------------|---------------------|
| 8.00     | 3.9  |                 | 0.15                |
| 8.10     | 4.9  | 0.010           |                     |
| 8.20     | 6.0  | 0.011           |                     |
| 8.30     | 7.2  | 0.013           |                     |
| 8.40     | 8.6  | 0.015           |                     |
| 8.50     | 10.1 | 0.016           |                     |
| 8.60     | 11.8 | 0.018           |                     |
| 8.70     | 13.7 | 0.020           |                     |
| 8.80     | 15.8 | 0.022           |                     |
| 8.90     | 18.1 | 0.025           |                     |
| 9.00     | 20.8 | 0.027           | 0.16                |
| 9.10     | 23.6 | 0.028           |                     |
| 9.20     | 26.4 | 0.029           |                     |
| 9.30     | 29.3 | 0.028           |                     |
| 9.40     | 32.1 | 0.027           |                     |
| 9.50     | 34.6 | 0.024           |                     |
| 9.60     | 36.9 | 0.022           |                     |
| 9.70     | 38.9 | 0.019           |                     |
| 9.80     | 40.6 | 0.016           |                     |
| 9.90     | 42.2 | 0.015           |                     |
| 10.00    | 43.7 | 0.014           |                     |
| 10.10    | 45.0 | 0.013           |                     |
| 10.20    | 46.2 |                 | 0.19                |

\*From H.A. McKenzie in Data for Biochemical Research, 2nd. ed. (1969), p. 495, ed. by R.M.C. Dawson et al. Reproduced with the permission of Oxford University Press.

change in absorbance which is associated with change in pH.

- 6 mM TRIS adjusted to pH 8.6 with HCl: absorbance = 0.39. Buffer absorbance can be suppressed. This buffer does not bind divalent metal ions such as Mg<sup>2+</sup>, Ca<sup>2+</sup> and Mn<sup>2+</sup>.
- 4 mM MES adjusted to pH 6.1 with NaOH: absorbance = 0.37. Buffer absorbance can be suppressed. This buffer has very little tendency to bind metal ions, and does not interfere with the Folin assay for proteins.
- 2 mM MES adjusted to pH 6.2 with NaOH and containing 0.1 M NaCl: absorbance = 0.31.
- 4 mM sodium citrate, pH 3.6: absorbance = 0.37.
- 1 mM HEPES adjusted to pH 7.3 with NaOH: absorbance = 0.27. The buffer does not bind Mg<sup>2+</sup>, Ca<sup>2+</sup>, Mn<sup>2+</sup> or Cu<sup>2+</sup>. The Folin protein assay cannot be performed in the presence of this buffer, but the Biuret assay is unaffected.
- 6 mM TAPS adjusted to pH 8.11 with NaOH: absorbance = 0.28. The buffer does not bind Mg<sup>2+</sup>, Ca<sup>2+</sup>, Mn<sup>2+</sup> or Cu<sup>2+</sup>.

#### De-salting (i.e. gel filtration followed by freeze-drying)

- 0.5% (NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub>, pH 7.8: absorbance = 0.24.
- 1 mM HCl, pH 2.9: absorbance = 0.015.
- 5 mM NH<sub>4</sub>OH, pH 9.6: absorbance = 0.07.

#### Solutions for cation-exchange chromatography

A concentration gradient of a salt is formed by mixing in





a varying ratio a buffer solution with the same buffer solution containing a high concentration of the salt.

1. Buffer solution — 0.025 N  $\text{H}_3\text{PO}_4$ , pH 2.3: absorbance = 0.002.  
High salt conc. — 0.5 M NaCl in 0.025 N  $\text{H}_3\text{PO}_4$ , pH 2.3: absorbance = 0.3.
2. Buffer solution — 5 mM acetic acid, pH 3.5: absorbance = 0.05.  
High salt conc. — 0.5 M NaCl in 5 mM acetic acid, pH 3.5: absorbance = 0.34.

### Solutions for anion-exchange chromatography

A concentration gradient of a salt is formed in the same way as for cation-exchange chromatography.

1. Buffer solution — 0.01 M  $\text{NH}_4\text{Cl}$  containing 100  $\mu\text{l}$  conc.  $\text{NH}_4\text{OH}$ /100 ml, pH 9.2: absorbance = 0.12.
2. High salt conc. — 0.25 M  $\text{NH}_4\text{Cl}$  containing 0.8 ml conc.  $\text{NH}_4\text{OH}$ /100 ml, pH 9.2: absorbance = 0.38.

In single-beam UV monitors such as LKB Uvicord S, the rising concentration of salt during ion-exchange experiments usually means a rising baseline on the UV record. This increasing absorbance can be compensated for by simultaneously running a counter-gradient of another substance which absorbs at 206 nm but which is otherwise "inert" in the system; for full details see Ref. 3, where acetone in concentrations up to 5.5% (v/v) was used to compensate for the rising baseline.

### Other buffer additives

1. 0.1% sodium dodecyl sulphate {SDS,  $\text{CH}_3(\text{CH}_2)_{11}\text{SO}_4^-\text{Na}^+$ } increases the solubility of slightly-soluble proteins: absorbance = 0.005.  
SDS-containing buffer — 0.1 M- $\text{NaH}_2\text{PO}_4$ , 0.1 M- $\text{Na}_2\text{HPO}_4$ , 0.1% SDS, pH 6.85: absorbance = 0.24.
2. 1 mM EDTA, pH 4.75 binds metal ions: absorbance = 0.11.
3. 8 M urea: absorbance = 1.48. Not suitable.
4. 1 mM 2-mercaptoethanol is used as a reducing agent for maintaining SH-groups in a reduced state: absorbance = 0.32.
5. 0.02% sodium azide ( $\text{NaN}_3$ ): absorbance = 1.6.  
0.05% merthiolate: absorbance = 0.7.  
0.0025% hibitane (chlorhexidine acetate): absorbance = 0.6.  
These three antimicrobial agents cannot, because of their high absorbance at 206 nm, be used in a

chromatography column *during an experiment*. However, it is quite permissible to add, for example, sodium azide to the buffer if the column is to be left unused for any length of time: before the column is used again, at least one bed volume of buffer free of sodium azide must be passed through it and a check made on the UV baseline.

### FINAL REMARKS

The buffers described and examined here are only examples, and it is of course possible to test the suitability of others for monitoring at 206 nm.

In those cases where the buffer must be diluted in order to obtain a reasonably low absorbance, the ionic strength may be considered very low. However, since the sensitivity of monitoring at 206 nm is so high, its primary use is for monitoring at low sample concentrations; hence the buffering capacity of the solvent need not be so high. One way to increase the buffering capacity of the solvent is to use a flow cell with an optical pathlength of 0.5 mm instead of 2.5 mm.

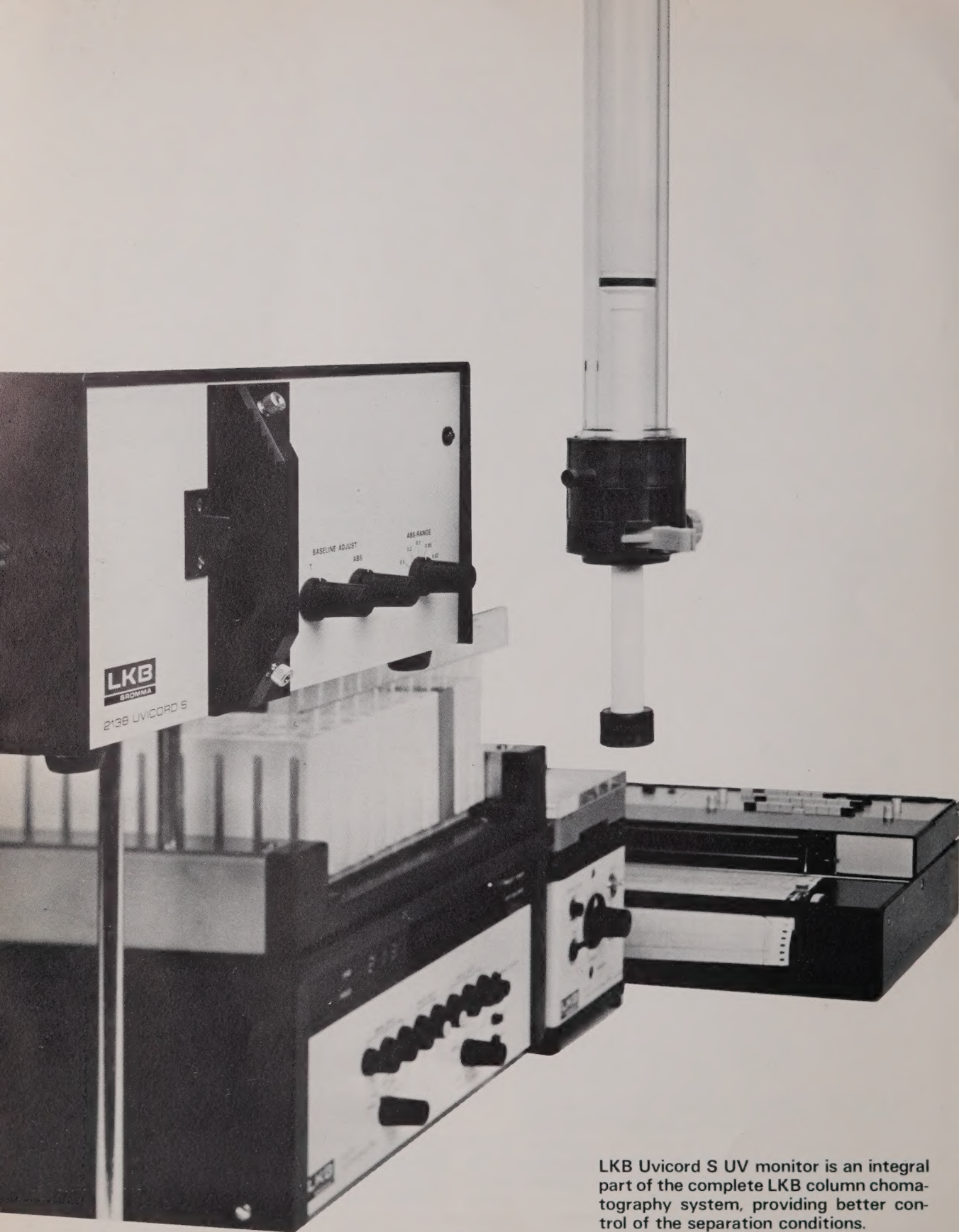
For example,

an absorbance of  $<0.4A$  is obtained with 4 mM MES/NaOH, pH 6.1, in a 2.5 mm cell and with 20 mM MES/NaOH, pH 6.1, in a 0.5 mm cell.

General precautions which should be taken when preparing buffers for monitoring at 206 nm are: only high-purity buffer components should be used; the water used should not contain residues from, for example, ion-exchange purification apparatus; and all glassware should be well cleaned and rinsed before use.

### REFERENCES

1. N.E. Good & S. Izawa in *Photosynthesis and Nitrogen Fixation, Methods in Enzymology*, Vol. XXIV, Part B (1972). Ed. by San Pietro, Academic Press, New York.
2. H.A. McKenzie, in *Data for Biochemical Research*, 2nd. ed. (1969). Ed. by R.M.C. Dawson et al., Oxford University Press, London.
3. J. Sjö Dahl, SCIENCE TOOLS in the press (1979).



LKB Uvicord S UV monitor is an integral part of the complete LKB column chromatography system, providing better control of the separation conditions.